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(54) Electrochemical detection of specific binding species

(57) The presence of an analyte in a sample, said analyte being a member of a specific being pair consisting of a ligand and an anti-ligand, is detected through the electrical signal produced by a chemical species with peroxidase activity which during the course of the assay becomes immobilised at or near a surface of the electrode. In particular, an immunoassay is based on the finding that peroxidase activity can be measured electrochemically at a polarised electrode when the peroxidase is immobilised to the electrode surface via an antibody-antigen bridge. The electrode may be carbon, graphite or a conductive polymer such as polypyrrole. The chemical species with peroxidase activity may be horseradish peroxidase, cytochrome c peroxidase, microperoxidase, fungal peroxidase or hemin derivatives such as protoferrihaem or deuteroferrihaem. The species may become immobilised on the electrode by reaction with a specific binding partner which is immobilised by adsorption or covalent bonding either to the electrode material or to one or more non-conductive materials that are intimately mixed with the electrode material.

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Electrochemical pseudo-homogeneous competitive binding assay for AhIgG

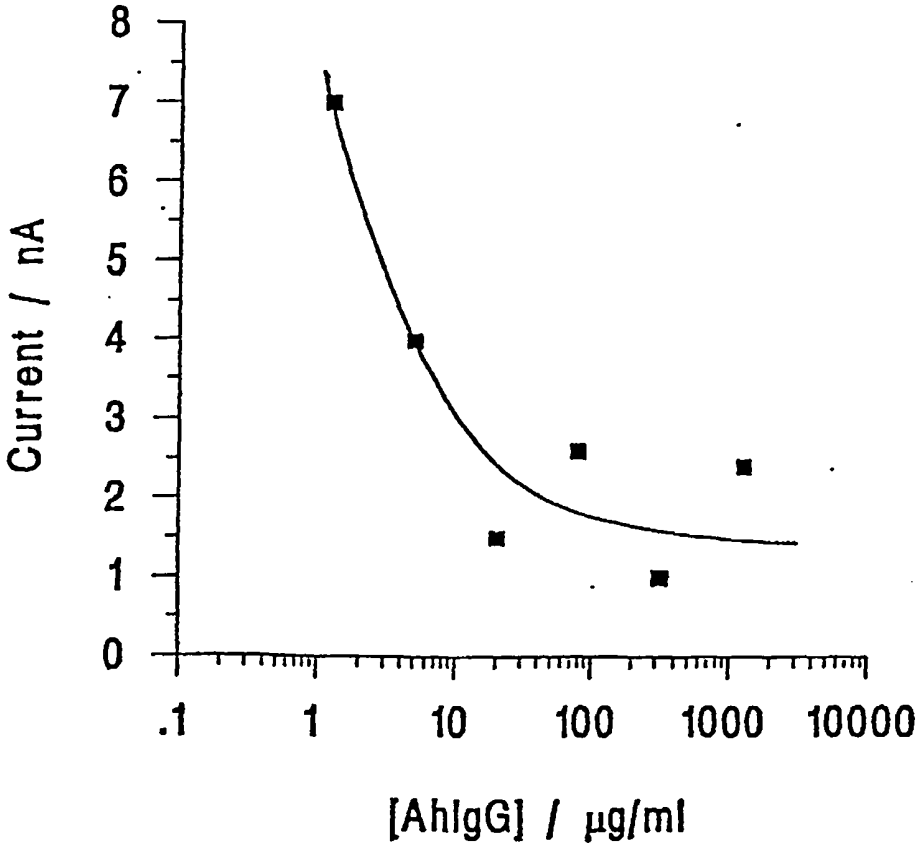

Fig. 1

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El ctrochemical Assay

Background of the Invention

This invention relates to a novel homogeneous immunoassay format. Immunoassays may be divided into 'homogeneous' and 'heterogeneous' categories. In a heterogeneous immunoassay, the analyte in the sample is used to bind a certain amount of a labelled species to a solid phase, via antibody-antigen interaction. A wash step then separates the bound label from the free label. One or another of these label populations may then be quantitated, the amount of label measured bearing a known relationship to the concentration of analyte in the sample. In a homogeneous immunoassay, no wash step is used: rather, binding of analyte in the sample to its specific binding partner (antigen or antibody) causes a change in the measurable activity of the label used. The advantage of the homogeneous assay is that it is easier to perform, requiring less operations than its heterogeneous counterpart.

In operation, however, those few homogeneous formats that have been demonstrated lack sensitivity, often because the change in activity of the label is not great. For example, one assay format known commercially as EMIT, from Syva, relies on binding of enzyme-labelled antigen to an antibody, in competition with the analyte: binding of the enzyme changes its specific activity, but only by a factor of approximately 50 %. Thus, there is a need for simple, homogeneous immunoassays in which the activity of the label undergoes a quantitative change.

A. L. Ghindilis et al., Biosensors & Bioelectronics, 7, 301-304 (1992), propose a potentiometric immunosensor based on the direct electrocatalytic electroreduction of oxygen in the presence of laccase. The laccase is conjugated to an antibody against insulin. The laccase-antibody is in competition with insulin in the solution and insulin immobilized on the electrode.

In the present invention, a novel homogeneous immunoassay format is described, based on the use of a peroxidase label which shows catalytic activity when immobilised to an electrode surface but although active in free solution it is not detected at the electrode. A peroxidase, such as horseradish peroxidase, normally catalyses the following reaction:

$$H_2O_2 + \text{ELECTRON DONOR} \longrightarrow \text{OXIDISED DONOR} + 2H_2O$$

where the electron donor is a soluble dye species, such as o-phenylendiamine (OPD) or tetramethyl benzidine (TMB). Recently, it has been discovered that horseradish peroxidase (HRP) can be immobilised to certain types of carbon electrode and, when polarised at a potential, such as 0 mV vs Ag/AgCl, can be made to reduce hydrogen peroxide in the absence of a soluble electron donor. Effectively, the electrode appears to take the place of the soluble donor. This effect has also been described for microperoxidase. It has been suggested that this phenomenon must be based on a direct electron transfer process and thus is critically dependent on intimate physical contact between the electron surface and the active site of the peroxidase enzyme (J Kulys and R D Schmid, Bioelectrochemistry and Bioenergetics 24 (1990), 305-311).

Summary of the Invention

A novel form of homogeneous immunoassay is described, using an electrode as the transducer and solid phase and using a peroxidase as the label (signal generating) species. It was found that peroxidase activity surprisingly can be measured at a suitable polarised electrode when the peroxidase is immobilised to the electrode surface via an antibody-antigen bridge, in the absence of a soluble electron donor species. This is surprising because previously it had been thought that in the peroxidase reaction an electrode can only replace the soluble

species as the electron donor if the peroxidase was physically adsorbed or directly immobilised to the electrode surface. The fact that peroxidase activity can be measured through a covering layer of protein (antibody and blocking agents) is unexpected and will mean revision of some theories currently in vogue as to the peroxidase/electrode so-called "direct electron transfer" process.

Furthermore, the peroxidase label when immobilised via an antibody bridge can be measured specifically even in the presence of peroxidase activity in the bulk solution. Thus the invention allows for the measurement of peroxidase conjugates in specific binding assays, such as immunoassays, without the need for a washing step to remove the unbound peroxidase conjugate or endogeneous contaminating peroxidase activity in the sample.

Detailed Description of the Invention

Methods and compositions are provided for determining an analyte that is a member of a specific binding pair. A specific binding pair is composed of a ligand and ligand receptor such as an antigen and antibody. One member of the specific binding pair is immobilised at or near the surface of an electrode; this may be termed "capture molecule". This may be done directly, via adsorption or covalent linkage to the electrode for example, or to another solid material intimately mixed with the electrode material; or it may be directly immobilised via a bridge molecule, for example an avidin/biotin bridge.

A member of the specific binding pair is conjugated to a signal producing means, said signal producing means having peroxidatic activity. Examples of such signal producing means are horseradish peroxidase, cytochrome c peroxidase, microperoxidase, ferrihaems such as deuteroferrihaem and other species undergoing the general reaction

$$H_2O_2 + A_{REDUCED} \longrightarrow A_{OXIDISED} + \text{other products}$$

and which on polarised electrodes can be made to undergo the reaction:

$$e^- + H_2O_2 \longrightarrow \text{other products}$$

in the absence of a soluble electron donor and where the electrons are donated by the polarised electrode. This signal producing means may be referred to as "molecule with peroxidase activity" and when conjugated to a member of a specific binding pair may be termed "peroxidase conjugate". The sample is incubated with the peroxidase conjugate and with the capture molecule (which may be immobilised already to the solid phase or may be free in solution, to be immobilised later).

By appropriate choice of specific binding pair components, an amount of peroxidase conjugate becomes immobilised at or near to the electrode surface that can be related to the concentration of analyte in the original sample.

The electrochemical assay according to this invention can be used in many different formats known to the man skilled in the art.

So it is possible to immobilise in any manner known to the man skilled in the art an antibody at the electrode surface for which the analyte to be measured and an analyte-peroxidase conjugate or an analyte analogue-peroxidase conjugate compete.

For a sandwich format a first antibody against the analyte to be measured is immobilised at the electrode surface and a second antibody labelled with a peroxidase is present in the solution.

In a competition format the solution contains a biotin labelled analyte or a biotin labelled analogue of the analyte. A peroxidase conjugate with avidin is also present in the solution or may be added after the capture reaction of the biotin

labelled analyte or analyte analogue and the analyte to be measured with the immobilised antibody. Other binding pairs in place of avidin/biotin, e.g. IgG-αIgG may be used equally.

It is also possible to use in a competitive assay an analyte or an analogue of the analyte conjugate with an anti-peroxidase-antibody and free peroxidase.

In a further competitive format the competition between the analyte and a peroxidase conjugate of the analyte or an analogue of the analyte with a peroxidase can be performed in a wick (bibulous layer) or a capillary channel in which an antibody against the analyte is immobilised on the surface. After having passed the wick or capillary channel the sample comes into contact with the electrode where an anti-peroxidase-antibody is immobilised.

Example

A pseudo-homogeneous competitive binding assay for rabbit anti-human immunoglobulin G was performed using a carbon electrode. The carbon electrode was supplied by E-Tek (Newton Highlands, Massachusetts, USA) and comprised a high surface area furnace black carbon (Vulcan XC-72, Cabot Corporation) bonded together with an equal weight of colloidal teflon (RTM) (PTFE) e.g. Fluon GPI, ICI), spread onto a graphite paper (Toray Industries, Japan) and sintered at 340 °C. A standard curve was constructed for the assay as shown in Figure 1. An electrode loaded with human immunoglobulin (hIgG) and blocked with milk protein, was used as the specific binding surface for a competitive binding reaction between free AhIgG and HRP labelled AhIgG. The incubation of each sample at room temperature was performed directly over the uncovered IgG-loaded electrode in the absence of a membrane. After incubation for 30 minutes, the electrode was polarised to +50 mV (vs Ag/AgCl) and a baseline response obtained. On

addition of 100 µM H_2O_2, the cathodic current change was measured for each sample.

A typical competitive assay curve for AhIgG is shown in Figure 1. The assay was performed without any separation steps or washing procedures.

Experimental

1. Human immunoglobulin G (hIgG, 10 µg/ml) was placed over an E-Tek electrode (1 cm^2) and incubated overnight at 4 °C.

2. The excess hIgG was removed by blotting and the electrode immersed in 1 % w/v milk blocking buffer [2.8 g dried skimmed milk (Marvel(RTM)) in TBS pH 7.4].

3. The electrode was rinsed briefly with PBS and a 2 mm electrode disk cut out and placed into the working electrode recess of a bowl cell containing a drop of 5 % hydroxyethylcellulose to effect electrical contact.

4. AhIgG (13 mg/ml stock) was diluted repeatedly (1:4) with 1 % milk blocking buffer to give a range of concentrations (2.5 - 2600 µg/ml).

5. HRP - AhIgG conjugate 1.3 mg/ml stock) was diluted with 1 % milk blocking buffer to 0.26 mg/ml. The AhIgG and HRP-AhIgG solutions were then mixed 1:1 thus having working concentrations of AhIgG and conjugate in the final mixture.

6. The AhIgG/conjugate (500 µl) was incubated over the hIgG/blocked electrode at room temperature for 30 minutes.

7. Without removal of the assay mixture, the electrode was polarised to +50 mV (vs Ag/AgCl) and a steady-state current baseline obtained with stirring.

8. 5 µl of a 10 mM H_2O_2 stock solution in PBS was added to the cell the final concentration of H_2O_2 being 100 µM, and the current response recorded.

9. After each measurement, the cell was rinsed with PBS and hIgG electrode disk replaced with another. The assay was repeated from step 6 using each AhIgG/conjugate mixture.

Claims

1. An electrochemical method for determining the presence of an analyte in a sample suspected of containing said analyte, said analyte being a member of a specific binding pair consisting of ligand and anti-ligand, wherein the amount of a detectable signal is a function of the amount of analyte in the assay medium, said detectable signal being detected at an electrode, wherein the detectable signal is produced by a chemical species with peroxidase activity which during the course of the assay becomes immobilised at or near a surface of the electrode.

2. A method according to claim 1 in which the chemical species with peroxidase activity is horseradish peroxidase, cytochrome c peroxidase, microperoxidase, fungal peroxidase, or hemin derivatives such as protoferrihaem or deuteroferrihaem.

3. A method according to claim 1 in which the electrode is a carbon electrode or graphite electrode.

4. A method according to claim 3 in which the chemical species with peroxidase activity becomes immobilised by reaction with a specific binding partner immobilised by adsorption or covalent binding to the carbon or graphite electrode.

5. A method according to claim 1 in which the electrode is formed from a mixture of carbon or graphite particles with chemical species that act to bind the carbon or graphite particles into a conductive electrode.

6. A method according to claim 5 in which the chemical species with peroxidase activity becomes immobilised by reaction with a specific binding partner that is adsorbed or covalently bonded to one or more nonconductive materials that is intimately mixed with the carbon graphite particles.

7. A method according to claim 1 in which the electrode comprises a conducting polymer.

8. A method according to claim 7 in which the conducting polymer is polypyrrole.

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Relevant Technical fields

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Search Examiner

D J MOBBS

Databases (see over)

(i) UK Patent Office

(ii) ONLINE DATABASES: WPI

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Documents considered relevant following a search in respect of claims 1-8

Category (see over)	Identity of document and relevant passages	Relevant to claim(s)
A	EP 0342382 A2 (GEC)	
A	EP 0125368 A1 (BATTELLE MEMORIAL INSTITUTE)	